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THE USE OF FLOW CYTOMETRY AND FLUORESCEIN-LABELED ANTIBODIES TO MEASURE SPECIFIC MILK PROTEINS IN BOVINE MAMMARY EPITHELIAL CELLS

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Summary

A flow cytometric technique was developed to measure the relative concentration of whey protein and β-casein in individual fixed and permeabilized bovine mammary epithelial cells. Primary bovine mammary epithelial cells were compared to mammary cells isolated from explants after a 24-h incubation and a bovine mammary epithelial transfected cell line (MAC-T). Cells were incubated with rabbit anti-bovine whey protein (α-lactalbumin + β-lactoglobulin) or β-casein primary antibodies followed by a fluorescein-labeled goat anti-rabbit IgG second antibody. The number and intensity of fluorescing cells were measured using an EPICS Profile Flow Cytometer. Primary and explant cells contained 3.3 and 2.8 times more whey protein than MAC-T cells. Explant epithelial cells contained 2.9 and 5.1 times more β-casein than primary or MAC-T cells. The higher concentrations of specific proteins within the cells was attributed to either greater synthesis or reduced secretion. These data show that flow cytometry is capable of detecting differences in milk protein concentration in different mammary epithelial cell types.

Key words: whey; β-casein; FITC; cell culture.

Introduction

Milk contains approximately 30 g casein and from 2.5 to 7.2 g whey protein/l whole milk (12,15,22). Current available methods for measuring specific milk proteins in mammary epithelial cells (i.e., radioimmunoassays, ELISA and gel electrophoresis) depend on the disruption of the cell's integrity (1,9,13,14,20). However, fluorescent microscopy has been used to detect specific milk proteins in intact cultured mammary cells microscopically (5,6,11). Also, flow cytometry has been used to measure DNA content (13,17), cell volume (4), and cell population dynamics (13,16).

Using flow cytometry to measure specific milk proteins in intact cells would allow for: (a) measuring the effects of various treatments on protein synthesis, (b) relating protein synthesis to DNA content and cellular differentiation, (c) sorting primary and transfected cells within a population on the basis of protein content, (d) following the health or productivity of a growing cell population, and (e) conducting numerous measurements on a given population of cells.

This report describes a flow cytometric assay using fluorescein-labeled antibodies to measure the relative content of whey and β-casein milk proteins within individual bovine mammary epithelial cells.

Materials and Methods

Cell preparation. Mammary tissue slices from healthy, mastitis-free quarters of mid-lactation Holstein cows were obtained at slaughter. The slices were washed and centrifuged twice in phosphate-buffered saline (PBS) to remove much of the milk adhering to the slices. The slices were then minced four times with a McIlwain tissue chopper (Mickle Laboratory Engineering Co., Ltd., Gomshall, Surrey, England). The epithelial cells were isolated nonenzymatically from tissue fragments by mixing the minced material in a 0.1 *M* PBS solution for 10 min and filtering the mixture through 30-μm nylon mesh. The filtrate containing the cells was centrifuged and the supernatant discarded. The cells were then fixed with a 70% ethyl alcohol:30% PBS solution as described (13) and stored at −20° C until prepared for cytometric analysis. Observation of the cell solution under a phase contrast microscope revealed whole cells and smaller pieces of debris as shown (13). Only the whole cells fluoresced when reacted with fluorescently labeled antibodies to casein, which is produced only by mammary epithelial cells (13). The small pieces of debris were discriminated against by the flow cytometer.

The mammary epithelial cells obtained by mechanical disruption of mammary tissue slices were compared to primary cultures of mammary epithelial cells and MAC-T (11) cells grown to confluence, cryopreserved in a freezing solution [70% culture medium, 20% fetal bovine serum (FBS), and 10% dimethyl sulfoxide] at −70° C and recultured as described (5). The primary cells stained positive for cytokeratin, milk protein, and esterase activity and negative for vimentin, which along with morphological examination established them as epithelial cells (5). Primary cells were grown to confluence in a modified Hanks' balanced salt solution (HBSS) described in (5) and then removed from culture plates with a trypsin-ethylenediaminetetraacetic acid (EDTA) solution. The cells were washed and centrifuged twice in HBSS, fixed with the 70% alcohol:30% PBS solution as described (13), and stored at −20° C until prepared for cytometric analysis. The use of 70% ethyl alcohol solutions to preserve cells was adapted from methods used to measure cellular DNA (3) and has been shown to preserve the properties of the target cell population with respect to relative DNA content (13).

Fluorescein labeling of milk proteins. Milk proteins within the cells were first tagged with rabbit anti-bovine α-lactalbumin + β-lactoglobulin (whey proteins) or β-casein (donated by Dr. J. Spies, Eastern Utilization, ARS, USDA) as the primary antibodies. Fluorescein isothiocyanate (FITC) labeled goat anti-rabbit IgG was used as the second antibody (Kirkegaard and Perry, Gaithersburg, MD).

Optimal dilutions for the primary and secondary antibodies were determined on cell suspensions containing approximately 100×10^3/ml of mammary epithelial cells prepared from explants and fixed in 70% ethyl alcohol. The alcohol was removed by centrifugation and the cell pellet incubated with

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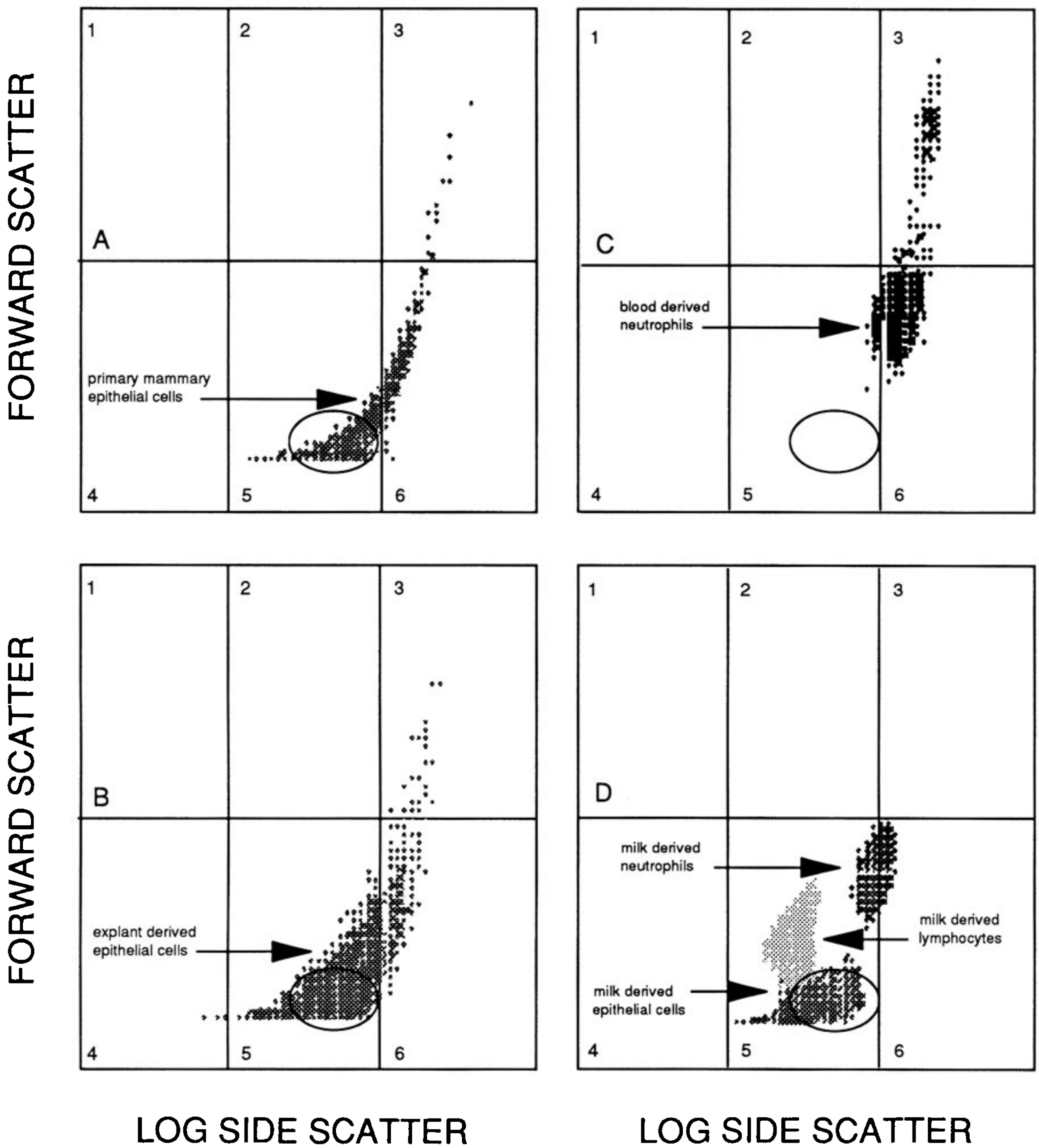

FIG. 1. Histograms used to separate cell types by size and shape utilizing forward scatter and log side scatter. *A*, Primary bovine mammary epithelial cells; *B*, bovine mammary epithelial cells isolated from tissue slices by mechanical disruption; *C*, neutrophils derived from blood serum; and *D*, neutrophils, lymphocytes, and epithelial cells in secretions obtained from a mastitis-infected quarter of a cow on Day 24 of her dry period. The *circle* drawn around the epithelial cell population in *D* is located at the same coordinates in *A*, *B*, and *C*.

1 ml of a 1:20 dilution of gamma-globulin-free horse serum in 0.01 *M* PBS containing 0.05% Tween 20 for 30 min at room temperature (RT). Tween 20 helps to permeabilize the cell walls and optimizes entrance of the antibody solutions into the cell. The suspension was centrifuged and the supernatant discarded. Both primary antibodies were diluted 1:10, 1:100, 1:1000, 1:10 000, 1:100 000, 1:1 000 000 with PBS and 500 μl added to the cell pellet. The cells were incubated at RT for 60 min with vortexing every 15 min, then centrifuged and washed three times with 500 μl PBS. The second antibody was diluted 1:50, 1:100, 1:200, and 1:400 when used with the primary antibody against whey protein, and diluted 1:20, 1:200, 1:2000, and 1:20 000 when used with the primary antibody against β-casein (Fig. 1), and then added to the cell pellet. After incubating at RT for 45 min with rocking, cells were washed three times with PBS. Then 250 μl PBS were added to the final pellet and placed on ice. The fluorescence intensity of individual cells was determined using the flow cytometric conditions described below. Rabbit preimmune serum and PBS were used as controls for nonspecific binding of rabbit antibodies.

Western blots. Specificity of the primary antibodies to whey and β-casein were tested with western blots (21). Total milk proteins from homogenized mammary tissue, purified bovine α-casein, β-casein, k-casein, γ-casein, α-

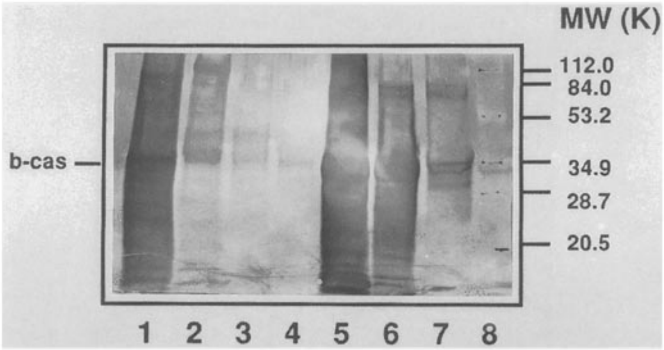

FIG. 2. Nitrocellulose western blot. *Lane 1*, bovine mammary tissue protein precipitant; *lane 2*, lysozyme + lactoferrin; *lane 3*, β-lactoglobulin; *lane 4*, α-lactalbumin; *lane 5*, β-casein (b-cas) + γ-casein; *lane 6*, β-casein; *lane 7*, α-casein; and *lane 8*, prestained standards treated with anti β-casein. Blotting was followed with alkaline phosphatase conjugated to a goat anti-rabbit IgG. Color development was with 5-bromo-4-chloro-3-indoyl phosphate and nitroblue tetrazolium.

lactalbumin (α-lac) and β-lactoglobulin (β-lac), lactoferrin, and lysozyme (Sigma Chemical Co., St. Louis, MO) were treated with Laemili buffer and run on a 12% acrylamide electrophoresis gel (13). The proteins were blotted onto nitrocellulose using an immun-blot assay kit (Bio-Rad Laboratories, Melville, NY) and treated with the primary antibodies for whey protein or β-casein. After washing, the blots were incubated with goat anti-rabbit IgG labeled with alkaline phosphatase. BCIP/NBT (Bio-Rad) was used as the alkaline phosphatase substrate.

Flow cytometric assay. The number of fluorescing cells and fluorescent intensity were measured using an EPICS Profile Flow Cytometer (Coulter Electronics, Hialeah, FL) equipped with a 488 nm argon ion laser. The fluorescent intensity of individual cells was measured through a 525 nm band pass filter in electronic channels from 0 to 1023. The higher the channel number, the greater the relative milk protein concentration per cell. Cells in channel 400 on a linear scale would have twice the concentration of a specific milk protein as cells in channel 200. Laser power was set at 15 mW. Single cells were separated from cellular debris and doublets on a forward scatter vs. log side scatter histogram and gated to a second histogram to measure log fluorescence. Mean channel of peak fluorescence was determined using Cytologic, a software program provided by Coulter.

RESULTS AND DISCUSSION

Identification of epithelial cells on flow cytometer. While epithelial cells overlap slightly with neutrophils and lymphocytes, as seen in Fig. 1 *D*, they do form a distinct population that can be isolated by bitmap gating. Histograms of epithelial cells derived from primary cell cultures (Fig. 1 *A*) and tissue slices (Fig. 1 *B*) that were used in this experiment depict populations of epithelial cells that do not appear to be contaminated by neutrophils (Fig. 1 *C*) or lymphocytes (Fig. 1 *D*).

Specificity of the primary antibodies. The specificity of the rabbit anti-β-casein primary antibody is shown (Fig. 2). The molecular weights of the caseins range from 19 000 to 25 000 but in the Laemmli gel system they migrate to the region near 30 000 (2). The anti β-casein antibody bound strongly to β-casein standards (*lanes 5* and *6*) and tissue extracted milk proteins (*lane 1*). There was some cross reactivity with the α-casein (*lanes 2–4* and *7*) but none with either β-lactoglobulin (*lane 3*) or α-lactalbumin (*lane 4*). This may represent α-casein contamination in the β-casein preparation used to immunize the rabbits to prepare the β-casein antibody. The commercial preparations of lysozyme + lactoferrin, β-lactoglobulin, and α-lactalbumin also appeared to contain α-casein and β-casein.

The specificity of the primary antibody to whey proteins (α-lac and β-lac) is shown (Fig. 3). The molecular weights of β-lac and α-lac are 18 400 and 14 300, respectively (2). The anti-whey protein antibody bound primarily to α-lac and β-lac as seen with the β-lac and α-lac standards (*lanes 3–4*) and the tissue extracted milk proteins (*lane 1*). The commercial standards used for β-lac, k-casein, β-casein, and α-casein (*lanes 3* and *5–7*) appear to contain a small concentration of α-lac and the α-lac standard (*lane 4*) appeared to contain some β-casein. There was faint binding to β-casein (*lanes 4* and *6*) but none to either k-casein or α-casein (*lanes 5* and *7*). This could be a consequence of the β-casein contamination in the whey protein preparation used to immunize the rabbits to prepare the whey protein antibody. There was strong binding to the lysozyme + lactoferrin standards (*lane 2*). While lysozyme + lactoferrin reacted with the anti-whey protein antibody in the intact cell, they did not appear in the tissue extracted proteins (*lane 1*).

Optimum antibody dilution rate. Peak binding of the primary antibodies occurred at a dilution of 1:100. Therefore, 1:100 vol/vol was used for the primary anti-whey protein and β-casein antibodies (Fig. 4). All four dilutions of the second antibody (1:50, 1:100, 1:200, and 1:400) used with the anti-whey protein primary antibody and the dilutions of 1:20 and 1:200 used with the anti-β-casein antibody demonstrated adequate binding (Fig. 4). The dilution of 1:200 vol/vol was chosen for the secondary FITC labeled goat anti-rabbit IgG antibody to have a single dilution for both primary antibodies (Fig. 4).

The degree of fluorescence or mean channel number is influenced by the Photo Multiplier Tube settings on the flow cytometer, the higher the setting, the higher the mean channel. A setting of 600 was chosen for the Photo Multiplier Tube so that the mean channels of cells with the lowest and highest milk protein content would be in the middle third of the mean channel scale. This prevented distortion of the mean channel that occurs when a histogram of cells with extremely high or low protein concentrations peaks at the upper or lower end of the mean channel scale.

Nonspecific and specific binding. The goat anti-rabbit second antibody showed minimal binding (mean channel of 71) to the primary mammary epithelial cells when PBS was used in place of the primary antibody (Fig. 5). When preimmune rabbit serum was used in place

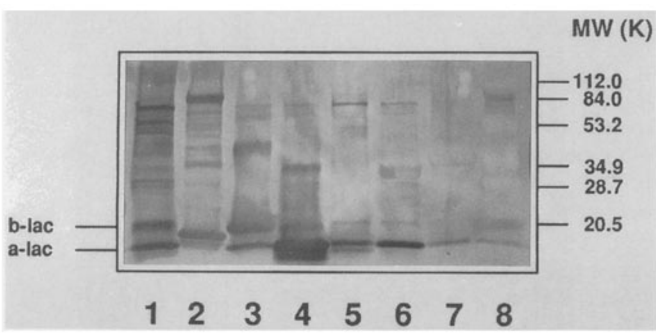

FIG. 3. Nitrocellulose western blot. *Lane 1*, bovine mammary tissue protein precipitant; *lane 2*, lysozyme + lactoferrin; *lane 3*, β-lactoglobulin (b-lac); *lane 4*, α-lactalbumin (a-lac); *lane 5*, k-casein; *lane 6*, β-casein; *lane 7*, α-casein; and *lane 8*, prestained standards treated with anti-whey. Blotting was followed with alkaline phosphatase conjugated to a goat anti-rabbit IgG. Color development was with 5-bromo-4-chloro-3-indoyl phosphate and nitroblue tetrazolium.

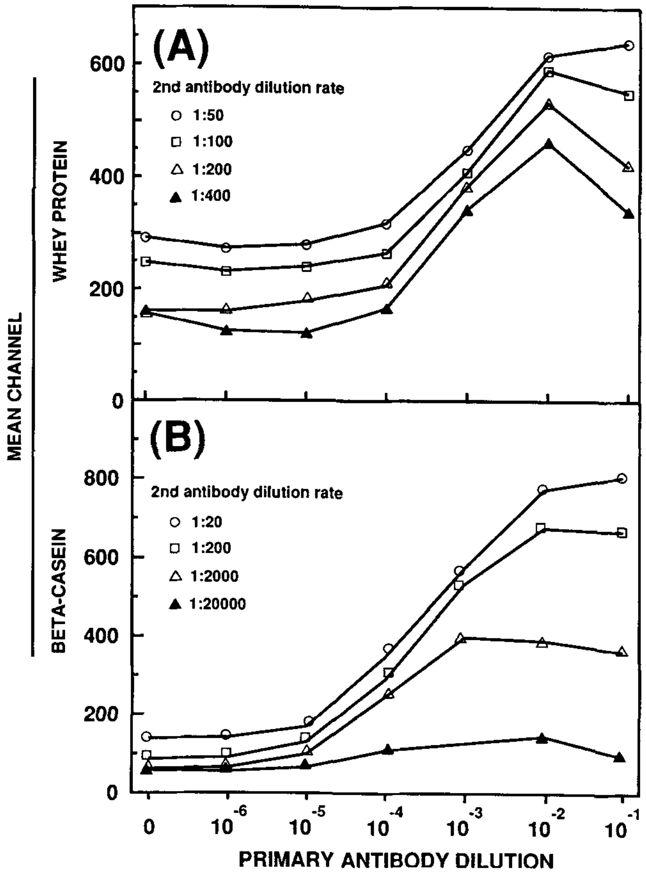

FIG. 4. The effect of antibody dilution rates on the mean fluorescence channel of bovine mammary cells. Primary rabbit anti-bovine whey (*A*) and anti-bovine β-casein (*B*) antibody dilution rates of 1:10, 1:100, 1:1000, 1:10 000, 1:100 000, 1:1 000 000 and phosphate-buffered saline (PBS) only are depicted on the X-axis. Secondary goat anti-rabbit IgG antibody dilution rates of 1:50, 1:100, 1:200, and 1:400 depicted by *lines* with either *open circles, open squares, open triangles*, or *closed triangles*, respectively, were used with the whey antibody as seen in (*A*). Secondary goat anti-rabbit IgG antibody dilution rates of 1:20, 1:200, 1:2000, and 1:20 000 depicted by lines with either *open circles, open squares, open triangles*, or *closed triangles*, respectively, were used with the β-casein antibody as seen in (*B*).

of the primary antibody, binding increased to a mean channel of 345. Binding increased to mean channels of 510 and 424, respectively, when the anti-whey and anti-β-casein were used as the primary antibodies (Fig. 5). Specific binding was determined by subtracting binding in the presence of rabbit preimmune serum from binding in the presence of the rabbit anti-whey protein or anti-β-casein sera. The flow cytometer was able to detect distinct differences between mammary explant cells, primary mammary epithelial cells, and MAC-T cells as seen in Table 1.

The failure of MAC-T cells, grown on collagen and supplemented with prolactin, to express whey protein or β-casein was attributed to their having been held at confluence for three weeks. Others (7,11) have demonstrated that MAC-T cells can produce casein and whey proteins when grown on collagen or mammary-derived matrix supplemented with prolactin.

Mammary explant cells contained greater amounts of β-casein and whey protein compared to the MAC-T cells. Explant cells also produced a greater amount of β-casein than primary mammary epithelial cells, but similar quantities of whey protein. This could be attributed to the short-term *in vitro* culture of the explants (24 h) as compared to multiple passages of the primary mammary epithelial cells. Others have shown that primary mammary epithelial cells can produce casein (18) and whey (19) proteins. These data suggest that explants more nearly represent *in vivo* conditions and are capable of producing and secreting casein (8,10,13) and whey protein (1,9,14).

However, the high cellular concentration of milk proteins in the explant cells could also have been due to proteins being synthesized but not secreted. Conversely, low cell concentration of proteins in primary epithelial cells may indicate that proteins were synthesized, but rapidly secreted into the medium.

Flow cytometry is a relatively easy method that does not require the disruption of the cell. These data show that flow cytometry can be used to measure the relative amounts of specific milk proteins in intact bovine mammary epithelial cells. Results also demonstrate that primary bovine mammary epithelial cells are capable of producing β-casein and whey protein.

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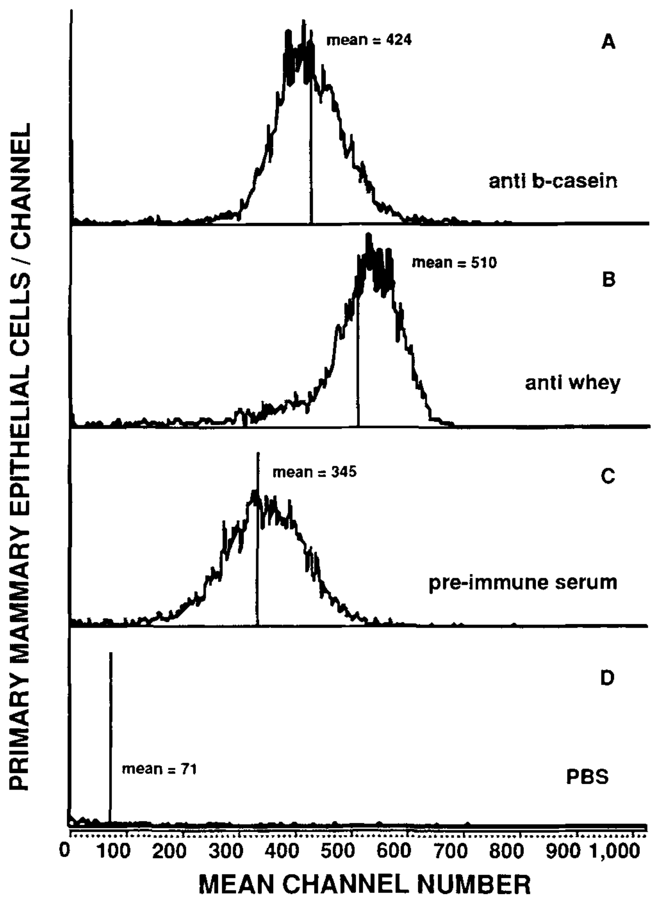

FIG. 5. Effect of rabbit preimmune serum on the fluorescence of primary bovine mammary epithelial cells. Cells incubated with rabbit anti-b-casein (anti-b-casein) (*A*), rabbit anti-whey protein (*B*), preimmune rabbit serum (*C*), or phosphate-buffered saline (PBS) (*D*) during the primary antibody step of the assay.

TABLE 1

RELATIVE CONCENTRATIONS (MEAN CHANNELS) OF WHEY AND β-CASEIN IN BOVINE MAMMARY EPITHELIAL CELL TYPES

	Mean Channel				
	Total		Nonspecific	Specific	
Cell Type	anti-Whey	anti-β-cas	Rabbit Preimmune	Whey-Preimmune	β-cas-Preimmune
Primary epithelial	510	424	345	165	79
Explant epithelial	535	575	345	190	230
MAC-T epithelial	403	390	345	58	45

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References

1. Akers, R. M.; McFadden, T. B.; Beal, W. E., et al. Radioimmunoassay for measurement of bovine α-lactalbumin in serum, milk and tissue culture media. J. Dairy Res. 53:419–429; 1986.
2. Basch, J. J.; Douglas, F. W.; Procino, L. G., et al. Quantitation of caseins and whey proteins of processed milks and whey protein concentrates, application of gel electrophoresis, and comparison with Harland-Ashworth procedure. J. Dairy Sci. 68:23–31; 1985.
3. Becton Dickinson. Monoclonal antibodies detecting cell proliferation and activation. Source book section 3.80:1–2; 1989.
4. Christensen, H.; Olsen, R. A.; Bakken, L. R. Flow cytometric measurements of cell volumes and DNA contents during culture of indigenous soil bacteria. Microb. Ecol. 29:49–62; 1995.
5. Cifrian, E.; Guidry, A. J.; O'Brien, C. N., et al. Bovine mammary teat and ductal epithelial cell cultures. Am. J. Vet. Res. 55:239–246; 1994.
6. Cifrian, E.; Guidry, A. J.; O'Brien, C. N., et al. Adherence of *Staphylococcus aureus* to cultured bovine mammary epithelial cells. J. Dairy Sci. 77:970–983; 1994.
7. Gazdag, A. C.; Hanigan, M. D.; Calvert, C. C., et al. Effects of mammary derived matrix on rates of milk protein production by the MAC-T mammary cell line. FASEB J. 6:A1116; 1992.
8. Gertler, A.; Cohen, N.; Maoz, A. Human growth hormone but not ovine or bovine growth hormones exhibits galactopoietic prolactin-like activity in organ culture from bovine lactating mammary gland. Mol. Cell. Endocrinol. 33:169–182; 1983.
9. Goodman, G. T.; Akers, R. M.; Friderici, K. H., et al. Hormonal regulation of α-lactalbumin secretion from bovine mammary tissue cultured in vitro. Endocrinology 112:1324–1330; 1983.
10. Hurley, W. L.; Aslam, M.; Hegarty, H. M., et al. Synthesis of lactoferrin and casein by explants of bovine mammary tissue. Cell Biol. Int. 18:629–637; 1994.
11. Huynh, H. T.; Robitaille, G.; Turner, J. D. Establishment of bovine mammary epithelial cells (MAC-T): an in vitro model for bovine lactation. Exp. Cell Res. 197:191–199; 1991.
12. Jenness, R. Comparative aspects of milk proteins. J. Dairy Res. 46:197–210; 1979.
13. Keys, J. E.; Van Zyl, J. P.; Farrell, H. M. In vitro DNA synthesis as indicator of mammary epithelial cell division: [^{14}C]thymidine uptake versus flow cytometry cell cycle analysis. In Vitro Cell. Dev. Biol. 30A:50–55; 1994.
14. Mao, F. C.; Bremel, R. D. Enzyme-linked immunosorbent assays for bovine α-lactalbumin and β-lactoglobulin in serum and tissue culture media. J. Dairy Sci. 74:2946–2951; 1991.
15. Mao, F. C.; Bremel, R. D.; Dentine, M. R. Serum concentrations of the milk proteins α-lactalbumin and β-lactoglobulin in pregnancy and lactation: correlations with milk and fat yields in dairy cattle. J. Dairy Sci. 74:2952–2958; 1991.
16. Nicoloso, F. T.; Val, J.; van der Keur, M., et al. Flow-cytometric cell counting and DNA estimation for the study of plant cell population dynamics. Plant Cell Tiss. Organ Cult. 39:251–259; 1994.
17. Shapiro, H. M. Practical flow cytometry. New York: Alan R. Liss, Inc.; 1988.
18. Talhouk, R. S.; Neiswander, R. L.; Schanbacher, F. L. In vitro culture of cryopreserved bovine mammary cells on collagen gels: synthesis and secretion of casein and lactoferrin. Tissue & Cell 22:583–599; 1990.
19. Talhouk, R. S.; Neiswander, R. L.; Schanbacher, F. L. Morphological and functional differentiation of cryopreserved lactating bovine mammary cells cultured on floating collagen gels. Tissue & Cell 25:799–816; 1993.
20. Thordarson, G.; Fielder, P.; Lee, C., et al. Mammary gland differentiation in hypophysectomized, pregnant mice treated with corticosterone and thyroxine. Biol. Reprod. 47:676–682; 1992.
21. Towbin, H.; Staehelin, T.; Gordon, J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedures and some applications. Proc. Natl. Acad. Sci. 76:4350–4354; 1979.
22. Vorherr, H. Human lactation and breast feeding. In: Larson, B. L., ed. Lactation. Vol. IV. Ames: Iowa State University Press; 1985:129–163.